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INTRODUCTION

Metastasis to distant organs is responsible for much of the morbidity and mortality of breast cancer. Recent research has focused on the host microenvironment as a target for new anti-cancer therapies [1, 2]. This research has shown that multiple host cell types contribute to tumor metastasis. However, little attention has focused on the neural component of the tumor microenvironment. Nerve fibers from the sympathetic branch of the autonomic nervous system innervate organs that are preferentially targeted by breast cancer metastasis, including lymph nodes, lungs, and bone [3-5]. By modulating the microenvironment targeted by metastasis, it is possible that the host sympathetic nervous system (SNS) may contribute to breast cancer metastasis in the presence of chronic stress.

Chronic stress can increase the density of SNS nerve fibers in lymph nodes by through the neurotrophic actions of Nerve Growth Factor [6]. This provides an anatomical basis for increased SNS signaling in this metastatic target tissue during periods of chronic stress. Furthermore, β-adrenergic receptors have been documented in multiple tumor types, including breast cancer [7-9]. *In vitro* studies have shown that SNS signaling regulates multiple pathways that converge on the metastatic phenotype, including tumor cell proliferation, invasion, angiogenesis, matrix metalloprotease activation, src oncogene signaling, and expression of interleukins-6 and -8 [10-12].

Chemokine signaling through the CXCR4-CXCL12 axis is one of the best characterized pathways involved in metastatic trafficking of breast cancer cells to distant tissues [13, 14]. Breast cancer cells frequently gain expression of the chemokine receptor CXCR4 during malignant conversion [15], which facilitates their directional migration toward its ligand CXCL12 (or stromal cell derived factor-1 [SDF1]) [16]. High levels of CXCR4 correlate with poor overall survival in breast cancer patients, and neutralization of CXCR4 reduces metastatic spread in a mouse model of breast cancer [17, 18]. It is plausible that chemokine receptor signaling via CXCR4-CXCL12 may be implicated in the effects of stress on metastasis. SNS signaling can up-regulate surface expression of CXCR4 on hemopoietic cells [19-22]. Furthermore, SNS signaling regulates transcription of CXCL12 in tissues targeted by metastatic breast cancer [23]. Given the key role of this chemokine pathway in breast cancer metastasis, these findings raise the possibility that stress-induced SNS signaling may regulate the dissemination and trafficking of metastatic breast cancer cells through the CXCR4-CXCL12 signaling pathway.

This project aimed to engage new imaging technology for non-invasive assessment of stress biology to identify the effects of SNS activation on the metastatic microenvironment, and evaluate the capacity of inhibitors targeted against the SNS or against SNS-mediated tumor mechanisms to block the effects of stress on breast cancer metastasis. The specific aims were to (a) define the magnitude and mechanism of sympathetic neural signaling pathways in breast cancer metastasis, and (b) to evaluate the effect on breast cancer metastasis of novel strategies that target either the SNS or down-stream molecular mechanisms that mediate SNS effects on metastasis.

REPORT BODY

Task 1. To investigate the effect of stress on spontaneous metastasis in a syngeneic, orthotopic model of breast cancer.

Objective: To define the effect of stress on breast cancer metastasis to clinically relevant organs.

1.1 Outcome summary: Quantitative and qualitative data on metastasis in control vs. stressed mice in a syngenic model of spontaneous breast cancer metastasis. **Overview:** To allow a sensitive assay of metastatic size, kinetics, and location, we established a system of *in vivo* bioluminescent tumor imaging in live mice. **Methods and Results:** To facilitate imaging, 66cl4 breast cancer cells were stably transduced with a lentiviral vector bearing firefly luciferase reporter gene. Luciferase assays showed that luciferase expression is stable in culture for >2 months, and for the duration of *in vivo* experiments (Figure 1A). At repeated study timepoints after tumor cell inoculation, tumor-bearing nude mice were injected with luciferin substrate, and luciferase-driven luminescence was detected by CCD camera (Figure 1A). Primary tumors are detectable by palpation at Day 7 after inoculation, but are already quantifiable by luminescence on the day of inoculation, which allows more sensitive analysis of tumor dynamics than previously possible. Metastasis to distant tissue sites, including axillary lymph node and lung, was detectable by luminescence at 20 days after inoculation (Figure 1A). **Conclusions:** The luciferase-tagged 66cl4 metastasis model recapitulates the homing properties of human metastatic breast cancer and facilitates real-time investigation of the trafficking dynamics that lead to metastatic colonization of distant tissues.

1.2 Overview: To assess the effect of chronic physiological stress on cancer progression, we used in vivo optical imaging to track metastatic homing of luciferase-tagged 66cl4 breast cancer cells from the mammary gland to distant tissues in immune-intact syngeneic Balb/c mice (Figure 1a,b). **Methods and Results:** Mice were randomly assigned to 2 hr/day restraint or home cage control conditions for 20 days, starting 5 days prior to tumor inoculation. Physical restraint is a well-established method of neuroendocrine activation that increases circulation of catecholaminergic neurotransmitters and cortisol (corticosterone), but avoids physical wounding that occurs in other behavioral stressors. Physiological stress response was confirmed in by 6.3% weight loss ($p = .004$) that was rapidly regained at the conclusion of restraint (Figure 1e). Chronic stress significantly increased metastasis to distant tissues, as shown by the significant Restraint x Time interaction in Figure 1b. In control mice, bioluminescent signal from metastatic tumor cells that had homed to the chest region (lung and lymph node) increased 10-fold over the first 3 weeks of tumor growth, plateauing at days 22-25 after primary tumor inoculation (Figure 1b, inset). Compared to controls, chronic stress increased the metastatic signal, resulting in a final metastatic signal >100-fold above baseline ($p < .001$) (Figure 1b). Stress-induced increases in metastasis occurred in clinically relevant tissues with 12.8-fold increase in lung ($p = .03$) and 3.0-fold increase in lymph node ($p < .001$) by ex vivo bioluminescent imaging of tissue-specific tumor signal (Figure 1a,c). Primary tumor growth, measured by caliper, was similar in restrained mice vs controls (Figure 1d). **Conclusion:** These results show that chronic stress can enhance spontaneous metastasis from an orthotopic primary tumor to distant tissues in a clinically relevant model. Through whole animal scanning to detect luciferase-tagged tumor cells, bioluminescence detection of tumor cells allowed repeated measures of primary tumor growth and metastasis in real time throughout the experiment, which obviated the need for sequential surgery or sacrifice in longitudinal studies.

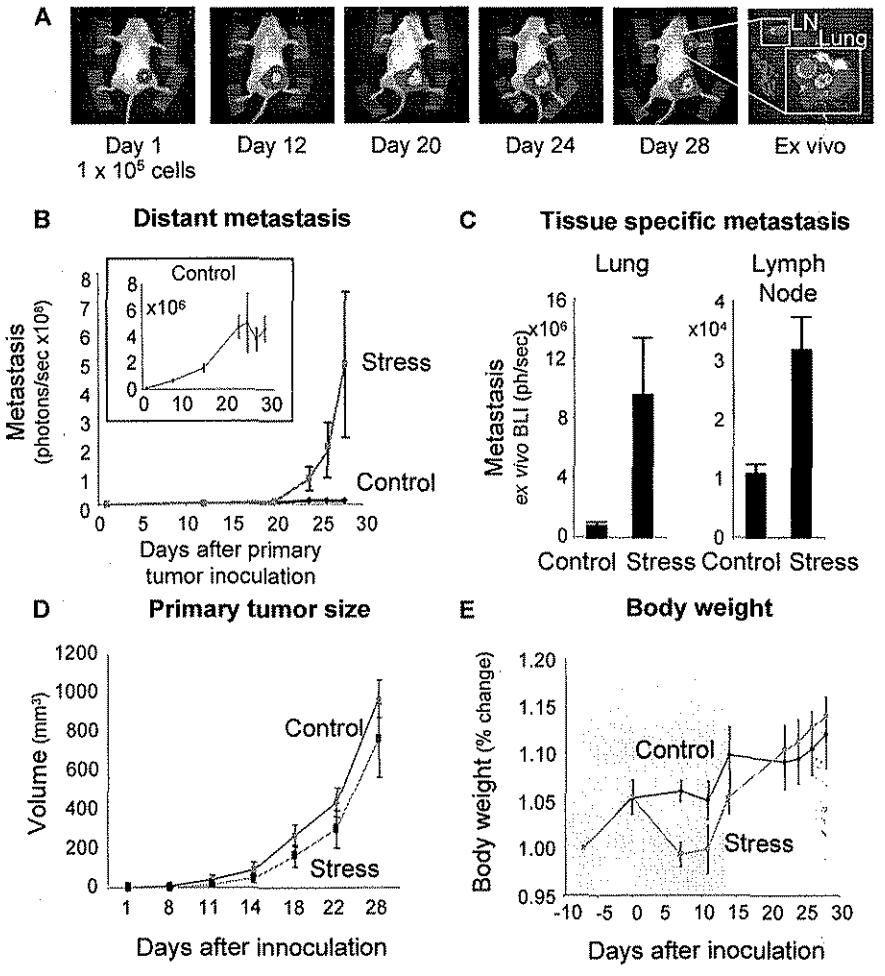


Figure 1. Chronic stress increased breast cancer metastasis to distant tissues. (A) Balb/c mice were inoculated into the 4th left mammary fat pad with 1 x 10^5 luciferase-tagged 66cl4 cells, and primary tumor, lymph node (LN) metastases, and lung metastases (Lung), were detected by repeated live *in vivo* bioluminescent imaging, and *ex vivo* imaging on Day 28. (B) Metastasis was quantified by repeated imaging of tumor-specific bioluminescence signal in chest region in live animals (average ± sem, control: black, restraint: grey). Inset shows control animals only, x axis: Time after tumor inoculation, y axis: metastasis (photons/sec x 10^6). (C) Ex vivo quantification of bioluminescent tumor signal in lung and axillary lymph node. (D) Primary tumor volume was determined by caliper measurements of tumor dimensions (average ± sem, control: black, restraint: grey). (E) Relative change in mouse body weight following tumor inoculation on Day 0 (average ± sem, control group: black, restraint group: grey, duration of stress: grey shading.

Task 2. To investigate the effect of stress on experimental metastasis in a human xenograft model of breast cancer.

Objective: To define the effect of stress on breast cancer metastasis in a model of human breast cancer.

Outcome summary: Quantitative and qualitative data on metastasis in control vs stressed mice in a xenograft model of breast cancer metastasis.

Overview: To assess the effect of chronic physiological stress on cancer progression in a xenograft model, we used in vivo optical imaging to track primary tumor growth and metastatic homing of luciferase-tagged MDA-MB-231 human breast cancer cells in immune-compromised SCID mice. **Methods and results:** SCID mice were inoculated into the 4th mammary fatpad with luciferase-tagged human MDA-MB-231 breast cancer cells. Half the mice were randomly assigned to chronic stress conditions by daily restraint (as in Task 1), and tumor growth and metastasis was tracked in real time using bioluminescent imaging (Figure 2a). Stress significantly increased growth of tumor cells in the orthotopic site (mammary gland) and metastasis to distant tissues as shown by increased tumor-specific bioluminescence (Figure 2b,c). **Conclusion:** Stress enhances growth of human breast cancer cells in the mammary gland, and leads to increased metastasis to distant tissues. Taken together with Task 1 findings, these results indicate that stress dynamics occur in the presence or absence of a functional lymphocyte compartment.

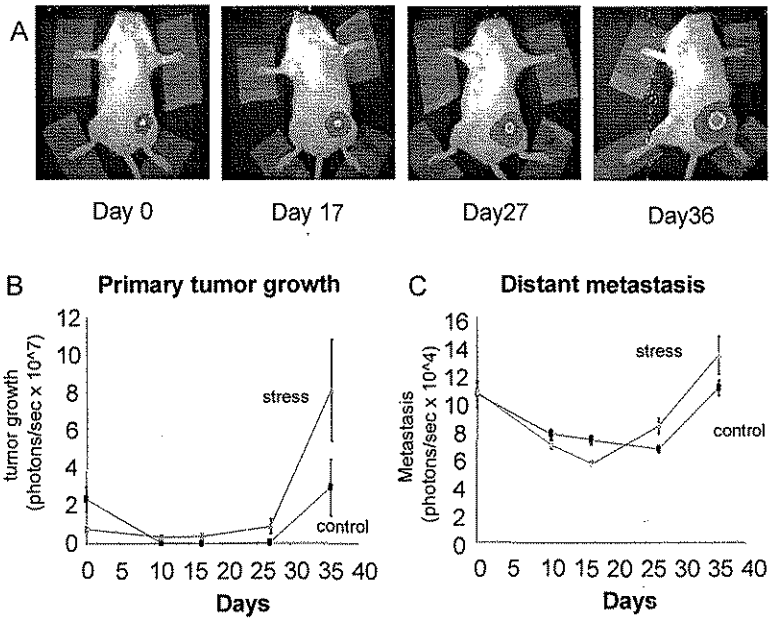


Figure 2. Stress enhances primary tumor growth and metastasis in a human xenograft model of breast cancer. A. Mice were inoculated into the 4th left mammary fat pad with 1 x 10^5 luciferase-tagged MDA-MB-231 cells, and tumor growth was tracked in real time by repeated live *in vivo* bioluminescent imaging. B. Primary tumor growth was quantified by repeated imaging of tumor-specific bioluminescence signal in live animals (average ± sem). C. Metastasis was quantified by repeated imaging of tumor-specific bioluminescence signal in chest region in live animals (average ± sem).

Task 3. To investigate the role of neural pathways in stress-effects on breast cancer metastasis by silencing nerve growth factor (NGF) neurotrophin activity in vivo.

Objective: To investigate the potential therapeutic value of silencing NGF expression as a potential treatment to inhibit the effect of stress on metastasis.

Outcome summary: In vivo delivery of siRNA was hampered by technical difficulties. However, the importance of neural pathways in stress-enhanced metastasis was demonstrated by pharmacological beta-blockade of sympathetic signaling.

Overview: This task aimed to investigate the potential therapeutic value of silencing NGF expression using directed siRNA to block stress effects on metastasis. Inefficient *in vivo* delivery of siRNA using liposomes resulted in insufficient knock-down of NGF expression to see biological effects. As an alternative method to investigate the potential of anti-metastatic therapeutic value of targeting stress-related neural pathways, we investigated the effect of pharmacological blockade of neural signaling using a beta-antagonist. Methods and results: Mice were inoculated into the mammary fatpad with luciferase-tagged 66cl4 breast cancer cells, and randomly assigned to control or stress conditions as in Task 1. In the context of a 2 (Control vs Stress) x 2 (Beta-blocker vs Placebo) study design, mice received either the beta-blocker propranolol or placebo in the form of a slow release pellet implanted the day before commencement of stress. Propranolol treatment did not alter stress-induced weight loss (Figure 3a) but inhibited stress-enhanced metastasis to distant tissues (Figure 3b,c). Conclusion: These results demonstrate that chronic stress enhances breast cancer metastasis through neural pathways. Given the central role of NGF in stress-induced neural activity [6], the finding that neural pathways play a central role in stress-enhanced metastasis suggests that further studies to investigate NGF activity are warranted. An alternative approach to blocking NGF function might make use of neutralizing anti-NGF immunotherapy to block stress effects on metastasis.

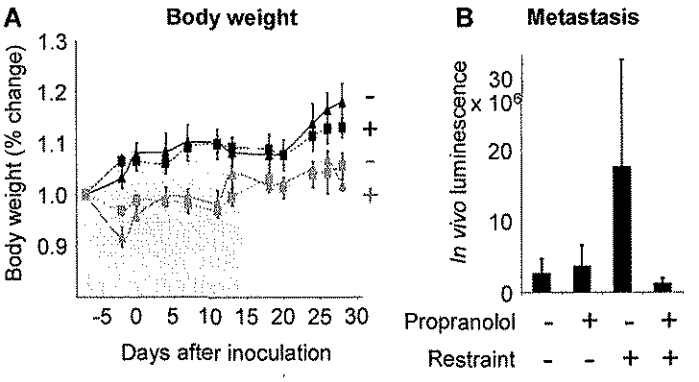


Figure 3. Beta-blockade abrogates stress-enhanced metastasis. (A) Effect of stress and beta-blockade on mouse body weight. Duration of restraint shown by shading (average ± sem, control group: black, restraint group: grey, placebo: solid line (-), propranolol: dotted line (+). (B) Metastasis was quantified by live *in vivo* imaging at 28 days after primary inoculation. (C) Representative images of mice in control, stressed (restraint) and stress + β-blocker groups. Primary tumor is the signal in the 4th right mammary fat pad (PT). Other foci are distant metastasis (M).

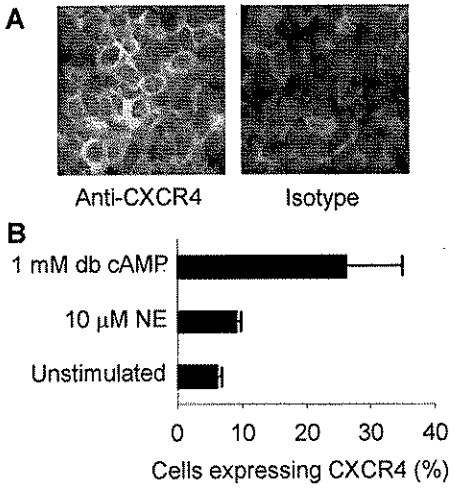
Task 4. To investigate the effect of anti-CXCR4 therapy on stress-enhanced breast cancer metastasis

Objective: To investigate the effect on stress-enhanced metastasis of blocking the chemokine receptor CXCR4.

Outcome summary: Stress effects were unexpectedly modified by the method of drug delivery (twice daily injections) which lead to reduced primary tumor growth. This was associated with reduced distant metastasis which prevented assessment of metastasis-specific effects of CXCR4 antagonism. Future studies of the role of chemokine signaling in stress-effects on metastasis are warranted and may involve modified (once daily) dosing protocol and use of experimental rather than spontaneous metastasis models.

4.1 Overview: To identify molecular mechanisms that might mediate stress-enhanced breast cancer metastasis, we examined SNS regulation of CXCR4 expression. **Methods and Results:** *In situ* expression of CXCR4 in primary 66cl4 tumors was assessed by immunostaining of fresh frozen tissues. Primary tumors express CXCR4 (Figure 4a) suggesting that these cells may be responsive to chemokine signaling. To determine the effect of SNS signaling on CXCR4 expression, 66cl4 breast cancer cells were treated overnight with 0 or 10 μM NE or 1 mM dibutyl (db) cAMP to mimic high physiologic levels of SNS signaling activation, and CXCR4 levels were assayed by flow cytometry. NE concentration was chosen to reflect levels found in epithelial tissues [24, 25]. Dibutyl cAMP concentration was chosen to reflect the amplification cascade as 10 μM NE ligates β2-adrenergic receptors and each receptor signals to multiple G-protein-adenylate cyclase complexes with rapid turnover of adenylate cyclase enzymatic activity [26]. The number of cells expressing CXCR4 increased by 50% after treatment with NE, and by 325% following treatment with db cAMP ($p < .01$)(Figure 4b). **Conclusion:** These data show that SNS signaling upregulates CXCR4 expression in 66cl4 breast cancer cells and suggests stress may enhance metastasis by increasing chemokine-directed homing.

4.2 Overview: To investigate the effect of chronic stress on chemokine-mediated trafficking of breast cancer cells *in vivo*, we examined the effect of CXCR4 inhibition on breast cancer metastasis in the context of a 2 (Stressed vs. Non-stressed) x 2 (CXCR4 antagonist vs. Saline Control) factorial design. Due to its ready availability and wide use in *in vivo* studies we chose to use the small-molecule inhibitor AMD3100. AMD3100 specifically targets CXCR4 to inhibit the binding and function of the chemokine CXCL12 with high affinity and potency [27]. **Methods and results:** Mice were randomly assigned to control or stress conditions as in Task 1 and luciferase-tagged 66cl4 breast cancer cells were inoculated in the mammary fatpad. Mice were randomized to received either the CXCR4 inhibitor or placebo by twice-daily subcutaneous injection commencing the day before stress. AMD3100 did not affect the rate of primary tumor growth. However, unlike in previous experiments (Figure 1), primary tumor growth was reduced by 2.0-fold in stressed mice vs. controls ($p < .001$)(Figure 4c). Reduced primary tumor growth may be related to additive effects of (i) restraint stress and (ii) increased handling that was associated with twice-daily delivery of drug/placebo. It is conceivable that this may have modified the stress effect, eg. through activation of alternative stress-response pathways such as the hypothalamic-adrenal-pituitary (HPA) axis. While Task 3 clearly showed the role of neural signaling in stress-enhanced metastasis, it is possible that HPA-related stress hormones such as corticosterone (cortisol) may suppress tumor growth in situations of extreme stress. Because of these unexpected suppressive effects on tumor growth, it was not possible to investigate the effect of AMD3100 on stress-enhanced metastasis. Future studies may make use of alternative method of drug delivery (eg. osmotic pump) or alternate method of pathway inhibition (eg. anti-CXCR4 immunotherapy) to prevent modulation of stress-responsive effects on mammary tumor growth. Alternatively, it may also be insightful to investigate the effect of CXCR4 signaling experimental metastasis models (i.e. tumor cell inoculation into blood stream). **Conclusions:** The role of CXCR4 signaling in stress-enhanced metastasis remains inconclusive. Further studies to investigate the role of CXCR4 signaling in stress-enhanced metastasis are warranted and should employ a modified study design that allows specific investigation of stress-related neural pathways.



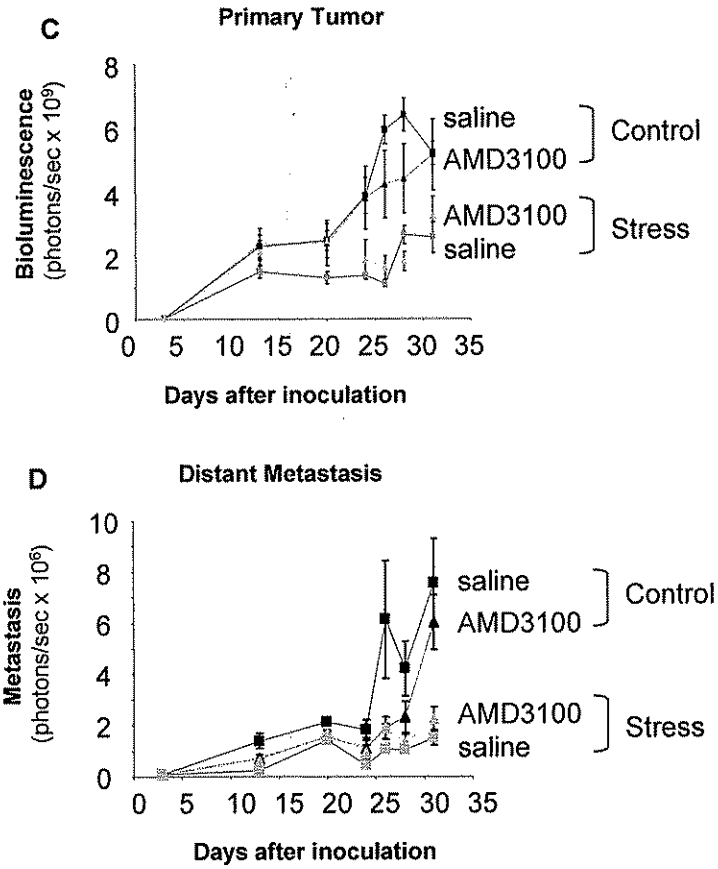


Figure 4. SNS signaling regulates chemokine receptor levels. A. 66cl4 primary tumors were stained with anti-CXCR4 antibody or isotype control. B. 66cl4 cells were stimulated with 0, 10μm NE, or 1 mM dibutyl (db) cAMP overnight, and CXCR4 levels were analyzed by flow cytometry. C, D. Primary tumor growth (C) and metastasis (D) were quantified by live invivo optical imaging.

KEY RESEARCH ACCOMPLISHMENTS

• Developed a sensitive in vivo assay using *in vivo* bioluminescent tumor imaging to reproducibly track stress dynamics with accurate measurement of metastatic size, kinetics, and location. The model recapitulates the homing properties of human metastatic breast cancer and facilitates real-time investigation of the trafficking dynamics that lead to metastatic colonization of distant tissues.

• Demonstrated that chronic stress increases the magnitude of distant metastasis from the primary mammary tumor by approx. 30-fold.

• Demonstrated that chronic stress enhances metastasis to clinically relevant tissues including lymph node and lung.
• Developed a xenograft model using MDA-MB-231 breast cancer cells that allows investigation of stress dynamics in realtime a human triple-negative (ER-, PR-, ErbB2-) breast cancer system.

• Demonstrated that chronic stress enhances both primary tumor growth and metastasis in a model of human breast cancer.

• Demonstrated that beta-blockade of sympathetic nervous system (SNS) activity completely inhibited stress-enhanced metastasis, showing that neural pathways are essential for stress-enhanced metastasis to distant tissues.

• Demonstrated that breast cancer cells express CXCR4 chemokine receptor and that SNS signaling increases CXCR4 expression on breast cancer cells, suggesting that chemokine signaling may play a role in stress-enhanced metastasis.

REPORTABLE OUTCOMES

• Presentations:
1. American Association of Cancer Research workshop on Pathobiology of Cancer. Title; Chronic peripheral neural activation increases distant metastasis from primary breast cancer. July 2009
2. PsychoNeuroImmunology Research Society Annual Conference. Title: Chronic stress enhances distant metastasis from primary breast cancer
3. Invited speaker at Peter MacCallum Cancer Centre Psycho-Oncology seminar, Melbourne, Australia. Title: Chronic stress and cancer

• The following data forms the basis of a manuscript in preparation. Expected submission: December 2009.

• Mouse models
Development of models to permit realtime analysis of stress-related tumor dynamics:
1. Immune-intact syngeneic mouse model of spontaneous metastasis from primary mammary tumor
2. Human xenograft model of spontaneous metastasis breast cancer metastasis from primary tumor

• Funding applications
Successful application to National Cancer Institute to further investigate the role of chemokine receptor signaling in neural regulation of breast cancer metastasis to distant tissues.

CONCLUSION

Tumor metastasis is the major cause of morbidity and mortality in breast cancer. The studies described here evaluated the translational opportunity of targeting the SNS as a common upstream regulator of multiple tumor-progression pathways that operate in the metastatic microenvironment. By exploiting sensitive imaging technology for non-invasive, real-time assessment of stress biology, these studies defined neural-associated mechanisms that operate in the tumor microenvironment to support homing and arrest of breast cancer cells in target organs. These studies identified chemokine receptor regulation as one potential molecular mechanism for the effects of chronic stress on metastasis. Further investigations of neural regulation of chemokine signaling in metastasis are warranted. These studies set the stage for future investigations of stress-effects on cancer progression, and suggest a novel therapeutic approach of targeting peripheral neural biology to protect stressed individuals from the adverse effects of stress biology on malignant disease.

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